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REVIEW

The *Francisella tularensis* Polysaccharides: What Is the Real Capsule?

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SUMMARY *Francisella tularensis* is a tier 1 select agent responsible for tularemia in humans and a wide variety of animal species. Extensive research into understanding the virulence factors of the bacterium has been ongoing to develop an efficacious vaccine. At least two such virulence factors are described as capsules of *F. tularensis*: the O-antigen capsule and the capsule-like complex (CLC). These two separate entities aid in avoiding host immune defenses but have not been clearly differentiated. These components are distinct and differ in composition and genetic basis. The O-antigen capsule consists of a polysaccharide nearly identical to the lipopolysaccharide (LPS) O antigen, whereas the CLC is a heterogeneous complex of glycoproteins, proteins, and possibly outer membrane vesicles and tubes (OMV/Ts). In this review, the current understanding of these two capsules is summarized, and the historical references to "capsules" of *F. tularensis* are clarified. A significant amount of research has been invested into the composition of each capsule and the genes involved in synthesis of the polysaccharide portion of each capsule. Areas of future research include further exploration into the molecular regulation and pathways responsible for expression of each capsule and further elucidating the role that each capsule plays in virulence.

KEYWORDS *Francisella tularensis*, capsule, lipopolysaccharide, O-antigen capsule, capsule-like complex, virulence, virulence factors

INTRODUCTION

Francisella tularensis is a Gram-negative, facultative intracellular bacterium responsible for the zoonotic disease tularemia (1). Tularemia is characterized by an acute onset of flu-like symptoms and granulomatous lesions of various tissues, including the

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lungs, lymph nodes, and spleen (1, 2). *F. tularensis* can be transmitted by arthropod bites, contact with infected tissues, laboratory exposure, and aerosolization (2). Additionally, *F. tularensis* infects numerous animals and invertebrates, which have the potential to become sources of infection for humans (3, 4). The various forms of tularemia—pneumonic, glandular, ulceroglandular, oculoglandular, oropharyngeal, and typhoidal—are highly dependent on the route of infection. Pneumonic tularemia is caused by inhalation of the bacterial cells and is considered the most serious form (2).

The disease severity of tularemia is also dependent on the infecting subspecies and subspecies clade. It has been well established that *F. tularensis* isolates can be divided into subspecies *tularensis* (Type A) and *holarctica* (type B) and that type A strains are more virulent than type B strains. However, molecular typing, particularly pulsed-field gel electrophoresis and whole-genome single nucleotide polymorphism typing, has been used to divide type A strains into clades of type A1a, A1b, and A2 (5, 6). Laboratory studies in mice and epidemiologic studies have shown that strains within clade A1b are more virulent than strains of clades A1a or A2 or type B (7–9). Furthermore, epidemiological studies have also shown that strains within clades A1a and A2 are no more virulent than type B strains (10). Geographically, type B strains are found worldwide across the Northern Hemisphere, whereas type A strains are concentrated specifically in North America (11).

The Centers for Disease Control and Prevention (CDC) considers both *F. tularensis* subspecies *holarctica* and *F. tularensis* subspecies *tularensis* tier 1 select agents due to their potential for use as bioweapons. *F. tularensis* has been classified as a potential bioweapon due to its virulence, ease of aerosol dispersal, persistence in the environment, and the nonspecific clinical signs of infection (12, 13). The World Health Organization estimated that a 10-kg aerosolized dispersal of *F. tularensis* could lead to 50,000 infections and possibly 4,000 deaths (2, 13). *Francisella novicida*, which by most experts is considered a distinct species (7), is not considered a significant threat to immunocompetent individuals and is not considered a select agent (14). No approved vaccine currently exists to prevent tularemia because the previously derived live vaccine strain, or LVS, is not considered safe and efficacious due to genetic instability, possible virulence to individuals with weakened immune systems, and a lack of protection against pneumonic infection (15).

Since the resurgence of tularemia research following the Amerithrax attack in 2001, many of the virulence properties of *F. tularensis* have been identified while investigators attempt to develop an effective vaccine (16). Well-characterized virulence factors include the *Francisella* pathogenicity island (FPI) that is essential for intracellular replication (17–19) and the unusual lipopolysaccharide (LPS) responsible for serum resistance and evasion of host defenses (15, 20–22). Other potential virulence factors of *F. tularensis* may include two different capsules: an electron-transparent O-antigen capsule (23) and an electron-dense capsule-like complex (CLC) (24, 25). Unfortunately, the common use of the term "capsule" for both components of *Francisella* has caused confusion, particularly in regard to previous literature on the subject.

Both Gram-negative and Gram-positive bacteria may produce capsules that function to primarily protect the bacterium from host defenses, particularly opsonization by antibodies to somatic antigens, complement-mediated killing, and resistance to phagocytosis (26–28). Specific antibodies to the capsule overcome these resistance mechanisms. Most capsules are polysaccharide polymers of two to three sugars and are negatively charged due to residues containing carboxylic acid, teichoic acid, phosphate groups, or other negatively charged moieties in their structures (29). However, the capsules of some bacterial select agents are atypical: the capsule of *Bacillus anthracis* is a polymer of the amino acid D-glutamic acid (30), and the capsule of *Yersinia pestis* is a protein polymer composed of 17-kDa subunits (31). The identity and characterization of a capsule, as it relates to *F. tularensis*, have not been clearly established until recently. This review aims to clarify and differentiate the characteristics of both capsules and to discuss the significance of these capsules and other polysaccharides of *F. tularensis* in relation to pathogenesis and virulence of the bacterium.

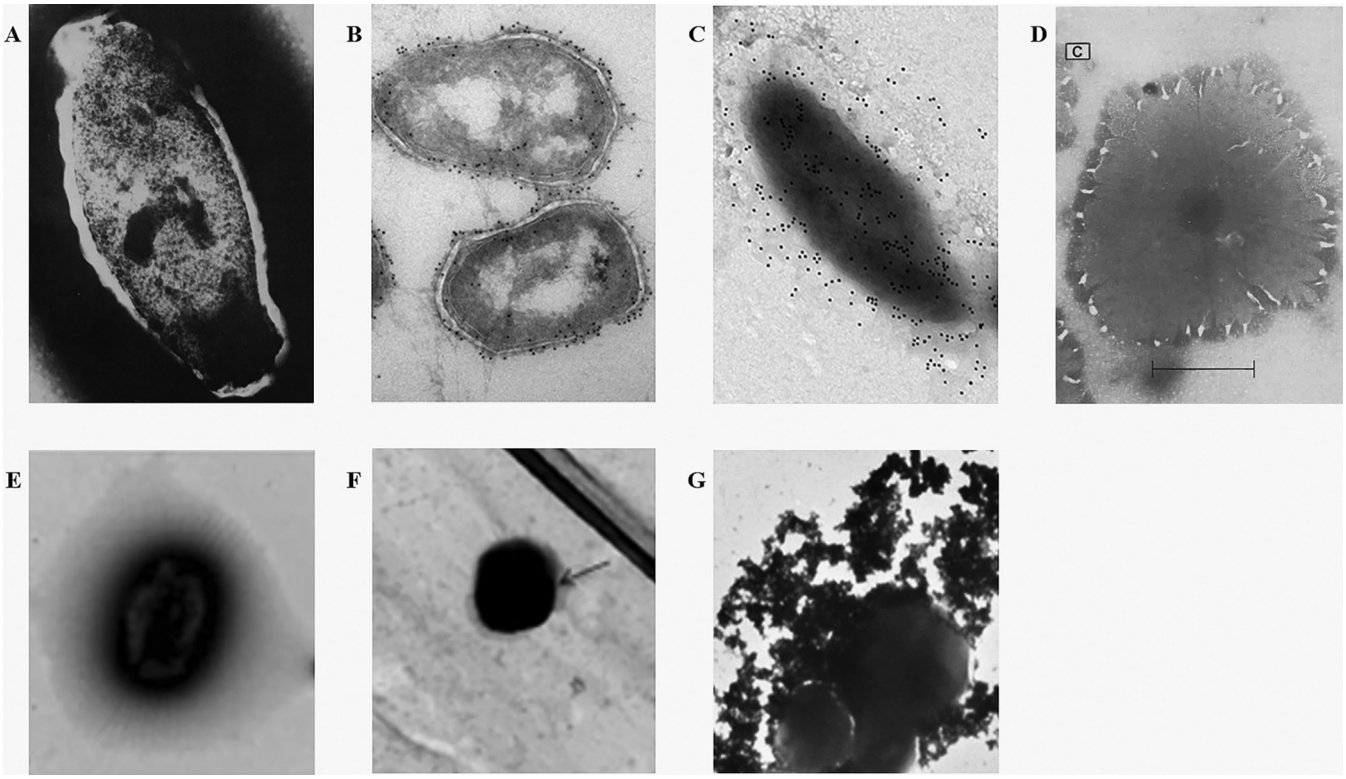

FIG 1 Transmission electron microscopy of the O-antigen capsule and the capsule-like complex (CLC) of *F. tularensis*. Both the O-antigen capsule and the CLC can be seen directly or indirectly surrounding *F. tularensis* cells by transmission electron microscopy. (A to C) The O-antigen capsule appears as a dark halo closely surrounding the cell when stained with phosphotungstic acid (A) or can be identified indirectly through binding of gold-labeled monoclonal antibodies specific to the O-antigen capsule in a cryo-immunoelectron micrograph (B) and a whole-mount immunoelectron micrograph (C). (D and E) The CLC appears as electron-dense material variably extending out from the cell after repeated subculturing in Chamberlain's defined medium. (F) LVS cells that have been grown on, but not subcultured in, defined medium exhibit only a small amount of CLC (arrow indicates possible CLC present). (G) In contrast to the CLC produced by LVS (D and E), the CLC produced by type A strains is more granular, appears to aggregate, and more closely resembles the CLC of *F. novicida* (Fig. 5A). (Panel A is republished from reference 34 with permission of the publisher; panels B and C are republished from reference 23; panel D is republished from reference 25 with permission from Elsevier; panels E and F are republished from reference from 24; panel G is republished from reference 54.)

THE ELECTRON-DENSE AND ELECTRON-TRANSPARENT CAPSULES

F. tularensis was first described by George W. McCoy as causing a "plague-like" disease of squirrels in Tulare County, California, in 1910 (32). With that discovery, work on creating an effective vaccine commenced. Many efforts were undertaken to find an immunogenic antigen conserved across *F. tularensis* strains to serve as a safe subunit vaccine. Immunodiffusion assays of extracted antigens from five *F. tularensis* strains indicated that a conserved extracellular polysaccharide consisting of four components existed between strains (33). Over a decade later, A. M. Hood (34) reported the first description and isolation of a capsule from *F. tularensis* SchuS4 in 1977. The capsule was described as being electron transparent and containing the sugars mannose and rhamnose and dideoxy sugars (34). When stained with phosphotungstic acid, the broth-grown bacteria were surrounded by a dark halo when viewed by electron microscopy (Fig. 1A). However, in 1994, Cherwonogrodzky et al. (25) reported that while LVS cells cultured from a frozen stock were nonencapsulated, following overnight growth in the minimal medium Chamberlain's defined medium (CDM), the presence of a capsule appearing as a dark to gray layer extending from and surrounding LVS cells (which we refer to as electron dense) was evident by electron microscopy. Following repeated subculture of the bacterium in CDM, this capsule-like material increased in size (Fig. 1D). This capsule is similar to the CLC reported around LVS cells after broth subculture in CDM and growth for several days at a lowered temperature on CDM agar (Fig. 1E). Prior work (25, 34) that is based on physical appearance and partial chemical analysis set the groundwork for further chemical and molecular characterization of

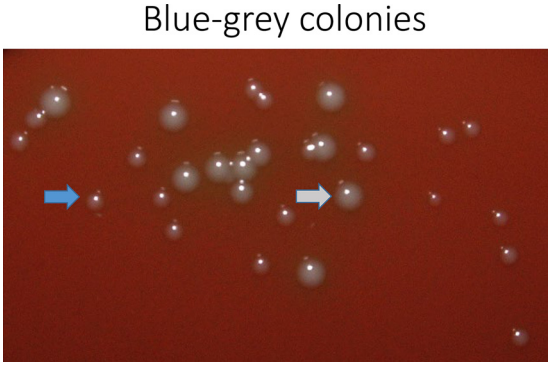

FIG 2 Blue and gray colonies of *F. tularensis* strain LVS. Upon subculture, *F. tularensis* can lose expression of the O antigen, resulting in loss of virulence. On blood agar, virulent *F. tularensis* colonies are smaller, with a blueish sheen (blue arrow). Spontaneous loss of the O antigen results in formation of larger, gray colonies (gray arrow) that are serum sensitive and avirulent in the mouse model.

these two separate capsules of *F. tularensis*: the electron-transparent O-antigen capsule and the electron-dense capsule-like complex (CLC). Figure 1 shows representative examples of the basic appearance of these two capsule types: Fig. 1A relies on the description of the O-antigen capsule by Hood (34); Fig. 1B and C show examples of the O-antigen capsule with subsequent immunolabeling (23) that better clarifies the extent of the O-antigen capsule; Fig. 1D and E offer examples of the CLC by Cherwonogrodzky et al. (25) and Bandara et al., respectively (24).

THE O-ANTIGEN CAPSULE

Discovery

One basis for classifying *F. tularensis* as being encapsulated is the demonstration of a change in colony phenotype after serial passage (35, 36) or application of various chemical mutagens (34, 37). Virulent *F. tularensis* grown on supplemented blood agar exhibits a small, smooth, white or blue opaque colony phenotype (Fig. 2, blue arrow) (35, 36, 38). Serial passage or chemical mutagenesis spontaneously leads to the appearance of *F. tularensis* colonies that appear larger, rougher, and gray (Fig. 2, gray arrow), and the bacteria are attenuated (35, 36, 38). Eigelsbach et al. used oblique lighting to classify colonies as blue or gray in heterogeneous cultures and showed that prolonged growth of a blue isolate leads to increased numbers of spontaneous gray colonies (36). Clinical manifestations of tularemia are also more severe, and the 50% lethal dose (LD_{50}) is much lower in mice inoculated with blue colonies than in those inoculated with gray colonies (36). This effect of apparent phase variation in colony phenotype and virulence has been shown repeatedly and can be attributed, in most cases, to the loss of the O antigen on the lipopolysaccharide (LPS) (15, 39). Prior to identifying the role of O antigen in this variation, this phenotypic change was still attributed to a change in surface polysaccharide components between strains (34, 35, 37). It is worth noting that the O-antigen locus is bordered up- and downstream by the transposase and pseudotransposase insertion sequences (IS) IS*ftu2* and IS*ftu1*, which may contribute to loss of O-antigen expression (Fig. 3) (40).

Characterization of virulent and spontaneous avirulent variants via electron microscopy documented the presence of an electron-transparent ring around the virulent strains (Fig. 1A) and the lack of such a ring around the avirulent variants (34). The presence of this electron-transparent ring also correlates with the colony phenotype and the ability of *F. tularensis* to resist the bactericidal effects of serum: gray colony variants that lack this electron-transparent capsule are sensitive to serum bactericidal activity whereas bacteria from blue colonies expressing O antigen are serum resistant (34, 37, 41). A crude extract of this capsular material contains mannose, rhamnose, dideoxy sugars, and fatty acids that differentiate the extracted material from components in the bacterial cell wall (34). Based on these findings, *F. tularensis* has been listed

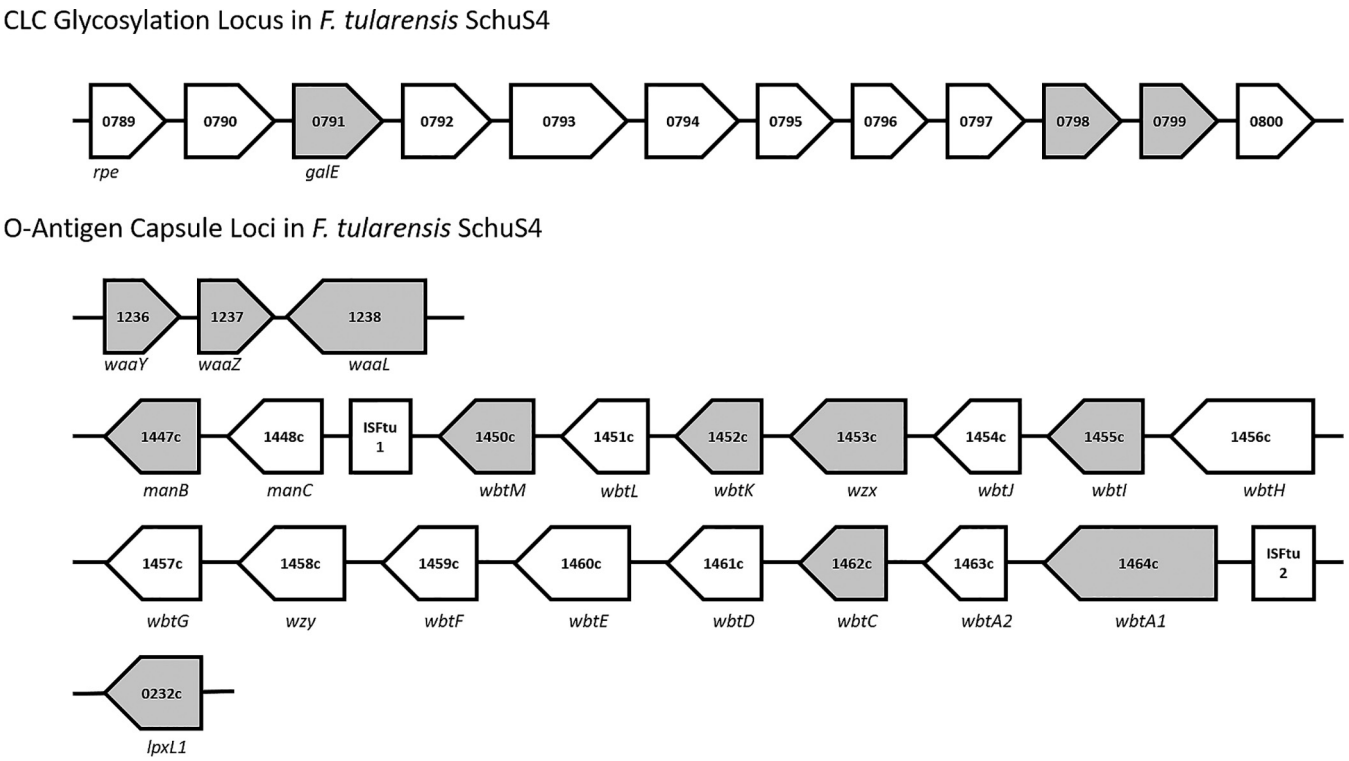

FIG 3 The CLC glycosylation locus and the O-antigen capsule loci of *F. tularensis* SchuS4. Locus tags and gene names, where known, responsible for glycosylation of the CLC and O-antigen capsule are listed. There are IS*ftu* insertion sequences immediately upstream and downstream of the locus responsible for lipopolysaccharide O-antigen synthesis.

as an encapsulated organism, but further isolation and characterization of this capsule were only recently completed in 2010 by Apicella et al. (23).

Further characterization of the O-antigen capsule was made possible by the production of monoclonal antibodies (MAbs) against a crude capsular extract (23) prepared in a manner similar to the extraction procedure described by Hood (34). Screening of these monoclonal antibodies against *F. tularensis* extracts identified a material that (i) is of large molecular size (>100 kDa) and diffuse in migration by Western blotting, (ii) is protease resistant, and (iii) precipitates in ethanol; these are hallmarks of a possible capsular polysaccharide. MAb 11B7 binds to this diffusely migrating large-molecular-size material in six *F. tularensis* strains that appears to match the aforementioned characteristics (23). MAb 11B7 also circumferentially labels *F. tularensis* cells, appearing to bind to a cell surface-associated antigen that is electron transparent, and the antigen could be identified by electron microscopy only when labeled with MAb 11B7 (23) (Fig. 1B and C). This material is effectively isolated from the crude extract through proteinase digestion, phenol extraction, Triton X-114 treatment, and chromatography (23). Compositional analysis of the material and reactivity with *F. tularensis* anti-LPS MAb FB11 indicate that this capsular polysaccharide is highly similar to the LPS O antigen (23). Further, *F. tularensis* mutants with disruptions in O-antigen synthesis genes do not produce this O-antigen capsule or the O antigen of the LPS, leading to the conclusion that this extracellular carbohydrate is a group 4 capsular polysaccharide or an O-antigen capsule (23). These results therefore clarify why acapsular mutants described previously are also O-antigen mutants and sensitive to killing by serum complement (37).

Composition and Structure

The O-antigen capsule appears to be conserved among *F. tularensis* strains and is composed of a 792-Da repeating unit identical to the subunit of the LPS O antigen (23). The O-antigen capsule carbohydrate is a repeating tetrasaccharide of 4)-α-D-GalNAcAN-(1→4)-

α-D-GalNAcAN-(1→3)-β-D-QuiNAc-(1→2)-β-D-Qui4NFm-(1 units (where Gal-NAcAN is 2-acetamido-2-deoxy-D-galacturonamide, QuiNAc is 2-acetamido-2,6-dideoxy-D-glucose, and Qui4NFm is 4,6-dideoxy-4-formamido-D-glucose), as determined by mass spectrometry and nuclear magnetic resonance analysis, and is identical to the chemical structure of LPS O-antigen (20). However, the O-antigen capsule is not fully identical to the LPS O antigen immunologically. The LPS O-antigen MAb FB11 binds both the O-antigen capsule and the LPS O antigen, whereas MAb 11B7 binds only the O-antigen capsule, indicating that there are differences in the epitopes of these two components (23). This difference in binding helps distinguish strains that produce LPS O antigen and not the O-antigen capsule. The O-antigen capsule also does not contain the inner core oligosaccharide that is present in the LPS. For example, 3-deoxy-D-*manno*-2-octulosonic acid (KDO), the first glycose that connects lipid A to the rest of the core oligosaccharide, was not detected in any capsular samples (23).

Additional metabolic labeling studies with [^{14}C]sodium acetate identified a lipid A-like molecule bound to the O-antigen capsule carbohydrate (42). This lipid is covalently bound to the O-antigen capsular polymers and can be separated from the carbohydrate by treatment with mild acid (42). The fatty acids present include three 3-OH fatty acids and one nonhydroxylated fatty acid and are grossly similar to the fatty acids present in the *F. tularensis* LPS lipid A structure (42). The ratio of the hydroxylated fatty acids differs between this lipid A-like structure, the LPS lipid A, and free lipid A. Both LPS lipid A and free lipid A have a ratio of greater than 5 to 1 of 3-OH-18:0 to 3-OH-16:0 fatty acids in contrast to less than 3 to 1 for the lipid-A like structure (42). The most prominent nonhydroxylated fatty acid in the lipid A-like structure is a $C_{14:0}$ (myristic acid), whereas palmitic acid ($C_{16:0}$) is found in the LPS lipid A and free lipid A (42). Further structural analysis of this lipid A-like structure has been hampered by the inability to purify larger quantities of the material.

Genetic Machinery

In addition to structure, the O-antigen capsule and the LPS O antigen share similar biosynthetic pathways (Fig. 3 and 4). The full biosynthetic pathway necessary for synthesis, assembly, and transport of the exported mature capsule has not been definitively defined, but general characteristics have been determined. In general, O-antigen glycosyltransferases (FTT1451c to FTT1464c) (Table 1 and Fig. 3) are responsible for the formation of the repeating tetrasaccharide of the O antigen for both the O-antigen capsule and LPS. Interruptions or deletions of the responsible genes in LVS, including *wbtI*, *wbtA1*, *wbtM*, and *wbtC*, result in loss of O-antigen capsule expression and a loss of O antigen on the LPS (23). Interruption of the gene *wzy*, responsible for an O-antigen polymerase, also disrupts the O-antigen capsule and LPS formation (43). Therefore, production of the O-antigen repeating unit appears to be through the same biosynthetic pathway regardless of the final product (i.e., O-antigen capsule or LPS). However, there appear to be differences in subsequent steps that differentiate the O-antigen capsule and the LPS O antigen. Waa proteins WaaY and WaaZ are likely responsible for the assembly of the core sugar onto lipid A, with WaaL mediating ligation of the O-antigen subunit to the core sugars on lipid A (44). Disruption of the responsible genes for the core oligosaccharide eliminates expression of the O antigen on the LPS and, in the case of WaaZ and WaaY, leads to a modified or truncated core oligosaccharide (43, 44). The O-antigen capsule is similarly disrupted in strains lacking WaaY or WaaL (43–45), indicating that a similar pathway may be used to ligate and export the capsule (Fig. 3). How these proteins specifically contribute to final production of the O-antigen capsule has not been determined. Proteins responsible for core sugar assembly and ligation likely play a role in producing the final, exported O-antigen capsule but do not play a role in assembly of the O-antigen subunits. These proteins may play a role in ligating the final O-antigen chain to a lipid A-like molecule prior to export of the capsule. Furthermore, mutants with a disruption of *wbtK* did not express the large-molecular-size O antigen but did express the O-antigen capsule (23). Thus, although

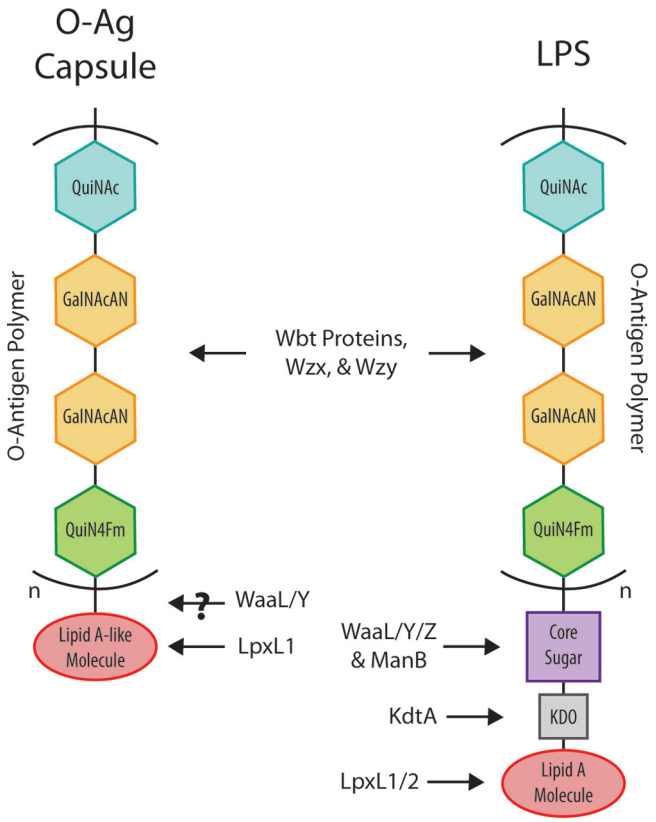

FIG 4 Comparative sketch structures of the *F. tularensis* O-antigen capsule and lipopolysaccharide. The polymers of the *F. tularensis* LPS O antigen and O-antigen capsule share the same tetrasaccharide repeating unit 4)-α-D-GalNAcAN-(1→4)-α-D-GalNAcAN-(1→3)-β-D-QuiNAc-(1→2)-β-D-Qui4NFm-(1- that is produced by the Wbt proteins, Wzx, and Wzy. Interruption of these proteins results in the loss of O-antigen laddering and the O-antigen capsule. The lipid A molecules of both the O-antigen capsule and LPS are assembled by Lpx proteins but differ in composition. Mutation of LpxL1 results in the loss of a lipid A subspecies for the O-antigen capsule but does not affect expression of the O-antigen capsule. *F. tularensis* strains with mutations in genes encoding proteins required for core sugar synthesis (WaaL, WaaY, WaaZ, and ManB) lack LPS O antigen. WaaL and WaaY are also required for expression of the O-antigen capsule, but the linkage between the capsule and the lipid is unknown, as indicated by a question mark.

most genes responsible for LPS O-antigen synthesis also contribute to O-antigen capsule expression, there are some genetic differences in production of the two antigens.

The late acyltransferase LpxL1 may also play a role in the export of a final O-antigen capsule (Fig. 3 and 4) (42). *F. tularensis* Lpx genes appear to be differentially controlled by temperature and have distinct fatty acid selectivity that accounts for the ability of *F. tularensis* to produce variable lipid A molecules (46). Barker et al. (42) note that interruption of LpxL1 leads to a reduction of a lipid A subspecies with remarkable similarities to the lipid A-like molecule of the O-antigen capsule (46). Mutation of the LpxL1 gene leads to the loss of the LPS O antigen but does not affect the O-antigen capsule (23). The O-antigen capsule lipid A-like molecule appears to require the LpxL1 gene for production. However, production of this lipid A-like molecule does not appear to be necessary for expression of the O-antigen capsule or transport of the O-antigen capsule.

Role in Virulence

The O antigen of LPS has long been understood to play a significant role in pathogenesis of *Francisella*. Lack of O antigen leads to serum sensitivity and loss of virulence for *F. tularensis* type A and B strains (15, 21, 43, 47, 48). All mutants lacking the O antigen had growth defects in cell culture and were attenuated in the mouse model

TABLE 1 Proposed genes involved in expression of the O-antigen capsule

F. tularensis subsp. *tularensis* SchuS4 ORF	*F. tularensis* subsp. *holarctica* LVS ORF	*F. novicida* ORF[b]	Gene name	Predicted protein product	Mutation reference(s)[a]
FTT_1236	FTL_0708	FTN_1254	*waaY*	Hypothetical protein	43–45
FTT_1237	FTL_0707	FTN_1255	*waaZ*	Glycosyl transferase family protein	43, 44
FTT_1238	FTL_0706	FTN_1256	*waaL*	Hypothetical protein	43–45
FTT_1447c	FTL_0609	FTN_1417	*manB*	Phosphomannomutase	43, 44
FTT_1450c	FTL_0606		*wbtM*	dTDP-D-glucose 4,6-dehydratase	23
FTT_1451c	FTL_0605		*wbtL*	Glucose-1-phosphate thymidylyltransferase	
FTT_1452c	FTL_0604		*wbtK*	Glycosyltransferase	23
FTT_1453c	FTL_0603		*wzx*	O-antigen flippase	43, 44
		FTN_1420	*wzx*	O-antigen flippase (*F. novicida* specific)	
FTT_1454c	FTL_0602		*wbtJ*	O-antigen protein	
FTT_1455c	FTL_0601		*wbtI*	Sugar transamine/perosamine synthetase	23
FTT_1456c	FTL_0600	FTN_1421	*wbtH*	Glutamine amido transferase/asparagine synthase	
		FTN_1422	*wbtN*	Glycosyl transferase	
FTT_1457c	FTL_0599	FTN_1423	*wbtG*	Hypothetical protein	
FTT_1458c	FTL_0598		*wzy*	Membrane protein/O-antigen protein	
		FTN_1424	*wzy*	Hypothetical protein	
FTT_1459c	FTL_0597	FTN_1425	*wbtF*	NAD dependent epimerase	
FTT_1460c	FTL_0596	FTN_1426	*wbtE*	UDP-glucose/GDP-mannose dehydrogenase	
FTT_1461c	FTL_0595	FTN_1427	*wbtD*	Galacturonosyl transferase	
		FTN_1428	*wbtO*	UDP-galactose phosphate transferase	
		FTN_1429	*wbtP*	Galactosyl transferase	
		FTN_1430	*wbtQ*	Aminotransferase	
FTT_1462c	FTL_0594		*wbtC*	UDP-glucose 4-epimerase	23
FTT_1463c	FTL_0593		*wbtA2* (*wbtB*)	Galactosyl transferase	
FTT_1464c	FTL_0592	FTN_1431	*wbtA1* (*wbtA*)	dTDP-glucose 4,6-dehydratase	23
FTT_0232c	FTL_0179	FTN_0072	*lpxL1*	LPS fatty acid acyl transferase	42

[a]References listed have analyzed production of the O-antigen capsule by strains with mutations in the specified genes.
[b]An ORF was not considered present in *F. novicida* if the similarity was not greater than 80% for the entire sequence of the ORF to the sequence of the LVS or type A ORF.

(23, 42, 44). Strains with mutations in *waaY* and *waaL* lack both the LPS O antigen and the O-antigen capsule, are more sensitive to complement-mediated lysis with serum, are phagocytized more readily, and are unable to replicate as efficiently as wild-type *F. tularensis* (45). These strains remain capable of disseminating to the liver and spleen after intranasal inoculation and are lethal to mice, but the mean time to death increases significantly compared to that of infection by the wild type (44). Histopathology of tissue samples from mice infected with *waaY* and *waaL* mutants also had a greater amount of gross inflammation, including necrosis, than those infected with the wild-type strain (44). Therefore, the O antigen of both the LPS and the O-antigen capsule may function as an immune-avoidance mechanism preventing the host from mounting inflammatory defenses.

Bear in mind that the differential contribution of the O-antigen capsule compared to that of the O antigen of the LPS to virulence and immune avoidance, if any, has not been elucidated. The two O-antigen components share similar biosynthetic pathways, and studies examining virulence have thus far utilized genetic knockouts or interruptions at shared points in the pathway. Differential growth studies have demonstrated that *F. tularensis* grown in brain heart infusion (BHI) broth is similar to host-adapted or macrophage-grown *F. tularensis* (49–51). Studies of the O-antigen capsule of *F. tularensis* grown in different media indicate, through antibody binding, that BHI broth-grown *F. tularensis* bacteria produce the greatest amount of O-antigen capsule and are the least accessible to antibodies directed at outer membrane components (51). When the bacteria are grown in Mueller-Hinton (MH) broth, which results in bacteria that are phenotypically distinct from macrophage-grown *F. tularensis*, less O-antigen capsule is produced, and there is an increase in binding of antibodies to outer membrane components (51). BHI broth-grown *F. tularensis* also stimulates a significantly lower tumor necrosis factor (TNF) and interleukin-1β (IL-1β) response from macrophages than MH broth-grown *F. tularensis*, where the Toll-like receptor 2 (TLR2) ligand lipoprotein Tul4A is readily

accessible (51). Collectively, these results suggest that the O-antigen capsule has a role in immune avoidance by preventing access of immune molecules to surface components of *F. tularensis*. Nonetheless, the differential roles of O-antigen capsule and LPS O antigen in virulence and resistance to host defenses are not clear. Virulence properties, such as serum resistance, that have been attributed to LPS O antigen may instead, or in addition, be due to the O-antigen capsule.

Passive and active immunization studies focused on the O-antigen capsule further support the contribution of this capsule to immune avoidance. Mice passively immunized with monoclonal antibody 11B7 survived a highly lethal intraperitoneal dose of LVS and did not develop clinical signs during the study period (23). A similar result was obtained when mice were immunized with two doses of purified O-antigen capsule and then challenged with virulent LVS (23). Additional studies focused on the type A strain indicate that the O-antigen capsule may be a necessary component to induce effective protection (52). Mice immunized with SchuS4 outer membrane preparations encapsulated in polylactic-co-glycolic acid (PGLA) followed by an intraperitoneal booster of LVS were partially protected from a virulent low-dose SchuS4 challenge (52). Western blot analysis using sera from the immunized mice indicated that the immunodominant antigen was the O-antigen capsule (52). This partial protection indicates that the O-antigen capsule alone may not be sufficient to induce a protective immune response against the more virulent type A strains of *F. tularensis* but may act as an adjunct in the design of a protective vaccine.

THE CAPSULE-LIKE COMPLEX

Discovery

Cherwonogrodzky et al. (25) investigated the effect of subculturing the live vaccine strain (LVS) on chemically defined medium to determine if such treatment would affect its virulence as previous work indicated that subculturing some bacterial species on a mildly acidic minimal medium enhances expression of their virulence factors (53). When *F. tularensis* was subcultured on Chamberlain's defined medium (CDM), the lethal dose in mice decreased, and the amount of negatively staining material surrounding the cells was increased, as shown by electron microscopy (25). Multiple passages of LVS in CDM greatly increased this material (Fig. 1D), and the colonies on agar became more mucoid, suggesting that this was capsular material whose expression was upregulated in this defined growth medium (25). This capsule was thought to be similar to the capsule described by other investigators based on electron micrographs (34, 37).

Around the same time that Apicella et al. (23) described the structure of the O-antigen capsule, Bandara et al. (24) isolated a separate capsule-like material from *F. tularensis* LVS. This "capsule" was produced and enhanced around LVS cells after multiple subcultures in CDM broth, followed by growth for 5 days on CDM agar at 32°C with 7% CO_2 (Fig. 1E). In contrast, overnight growth on CDM agar from a frozen stock resulted in only a small amount of CLC-like material on the cell surface (Fig. 1F). Interruption of genes within a novel glycosylation locus (Fig. 3, FTT_0789 to FTT_0800) abolished production of this capsule (24). This material was different from the O-antigen capsule in appearance, carbohydrate composition, and genetic machinery. Termed the capsule-like complex, or CLC, the isolated material contained the sugars mannose, galactose, and glucose and could be isolated from strains completely lacking O antigen, both on the LPS and as a capsule (24). The CLC was therefore a separate entity from the O-antigen capsule.

Composition and Structure

The CLC is a heterogeneous complex of protein and carbohydrate (24) whose expression is enhanced by subculture on chemically defined medium and growth at a temperature lower than 37°C (24, 25, 54). The material was first isolated from O-antigen mutants of *F. tularensis* LVS serially subcultured on defined medium so there was no contamination by LPS O antigen (24). The CLC was initially extracted with 0.5% phenol, followed by purification using enzymatic digestion, ethanol precipitation, and ultra-

centrifugation (24). Electrophoretic separation of the material revealed a large array of variously sized proteins and the presence of a high-molecular-weight (HMW) carbohydrate with no evidence of LPS or O-antigen contamination of the extracts (24). The CLC extracts do not react with the O-antigen capsule monoclonal antibody 11B7, and an identical material can be extracted from the O-antigen-negative LVS with a G197V substitution in WbtI (LVS $WbtI_{G197V}$), further differentiating the two materials (24, 54). At least some of the proteins present in the CLC are proteinase K resistant, and approximately 10% of the material is a combination of mannose, glucose, and galactose residues (24). However, when sedimented by ultracentrifugation or concentrated by ultrafiltration, the CLC becomes highly insoluble and cannot be resolubilized, preventing further resolution of the components (24). Bandara et al. (24) postulated that the complex material contained multiple glycoproteins and an HMW carbohydrate. Similar to the O-antigen capsule, the CLC appears to be conserved between *Francisella* type A and type B strains as both LVS and SchuS4 express CLCs with similar or identical electrophoretic profiles (54).

To improve solubility of the CLC, alternative extraction media were examined, resulting in an extraction protocol utilizing 1 M urea in place of phenol and no proteinase K treatment. To further improve solubility, the detergent Triton X-114 was used for some extractions. These modifications resulted in extracts with electrophoretic profiles that were similar to those of the 0.5% phenol extracts but were more water soluble and amendable to further analysis (54). Size fractionation of this urea-extracted material using a GelFree fractionation unit revealed that the CLC is mainly composed of proteins or glycoproteins less than 150-kDa in size; the HMW band of greater than 150-kDa is no longer present (54). As a result, Champion et al. (54) postulated that the previously observed HMW band is an aggregate of glycoproteins and proteins that disassociated during size fractionation. Based on mass spectrometry analysis, the CLC is composed of mainly acidic or hydrophobic amino acids that likely contribute to the aggregation and insolubility of this complex prior to fractionation (54). Analysis of in-gel digests of these proposed aggregates prior to fractionation identified a 420-Da subunit that appears to be glycan in nature (54).

Following fractionation of the CLC, a 45-kDa protein is the most reactive component to anti-CLC hyperimmune serum by Western blotting (54). This protein is similar in size to a 45-kDa protein described by Huntley et al. (55) that is reactive with whole-cell lysates of LVS and SchuS4 following immunization of mice with LPS. This unidentified protein could be FopA, a highly immunogenic 43-kDa outer membrane protein (56) that has been shown to be glycosylated (57). FopA is also present in outer membrane vesicles and tubes (OMV/Ts) produced by *F. novicida* (58). Further electron microscopic analysis and mass spectrometry suggest that the CLC may in part be, or contain, OMV/Ts as the CLC shares the gross similar appearance and composition of the described OMV/Ts. In total, 68 proteins have been identified in LVS CLC extracts, as determined by nano-liquid chromatography-mass spectrometry of trypsin digests; 56 proteins are present in the insoluble portion of the extract, and 12 proteins are present within the soluble portion (54). However, many of the proteins are heavily glycosylated and cannot be identified (54). Of the identified CLC proteins, 8 of the 12 soluble proteins and 38 of the 56 insoluble proteins are present in *F. novicida* OMV/Ts (58, 59). However, the OMV/Ts described by Champion et al. are from LVS cells enhanced to express CLC or are from LVS mutants, but their appearance and protein composition are comparable to those of OMV/Ts from wild-type *F. novicida* (54). The appearance and composition of OMV/Ts from wild-type type A or type B cells have not yet been determined. Isolation and identification of proteins within type A or B OMV/Ts will be necessary to determine the exact extent of overlap between the CLC and the OMV/Ts. At this time, definitive determination of whether the CLC is purely enhanced expression or overproduction of OMV/T or if OMV/Ts are a separate entity that have associated with the CLC cannot be made.

The HMW band of the CLC may be the same HMW carbohydrate smear observed by Zarrella et al. following growth of LVS in BHI broth (51). This HMW carbohydrate is a

TABLE 2 Proposed genes involved in expression of the CLC

F. tularensis subsp. *tularensis* SchuS4 ORF	*F. tularensis* subsp. *holarctica* LVS ORF	*F. novicida* ORF[b]	Gene name	Predicted protein product	Mutation reference(s)[a]
FTT_0789	FTL_1432	FTN_1221	*rpe*	D-Ribulose-phosphate 3-epimerase	
FTT_0790	FTL_1431	FTN_1220		Glycosyl transferase	
FTT_0791	FTL_1430	FTN_1219	*galE*	UDP-glucose 4-epimerase	60
FTT_0792	FTL_1429	FTN_1218		Glycosyl transferase	
FTT_0793	FTL_1428	FTN_1217		ATP-binding membrane transporter	
FTT_0794	FTL_1427			Hypothetical protein	
FTT_0795	FTL_1426			Hypothetical protein	
FTT_0796	FTL_1425			Hypothetical protein	
		FTN_1216		Hypothetical protein with methyl transferase domain	
		FTN_1215		Capsule polysaccharide export protein	
FTT_0797	FTL_1424	FTN_1214		Galactosyl transferase	
FTT_0798	FTL_1423	FTN_1213		Galactosyl transferase	24, 60
FTT_0799	FTL_1422	FTN_1212		Mannosyl transferase	24
FTT_0800	FTL_1421	FTN_1211		Haloacid dehalogenase	

[a]References listed have analyzed production of the CLC by strains with mutations in the specified genes.
[b]An ORF was not considered present in *F. novicida* if the similarity was not greater than 80% for the entire sequence of the ORF to the sequence of the LVS or type A ORF.

diffusely stained region that extends above 225 kDa, is protease resistant, and is labeled with both carbohydrate and protein stains (51). The profile of the HMW carbohydrate greatly resembles the profile of the CLC prior to fractionation; however, confirmation that the HMW and CLC are the same entity has not been made. Nonetheless, a second putative glycan fragment was observed in soluble portions of the CLC that contains the HMW carbohydrate (54). This hexasaccharide is composed of monosaccharide units in the sequence (Da) 203-223-203-162-162-203, with the 203-Da moiety possibly representing *N*-acetylhexosamine (HexNAc), the 162-Da unit possibly representing an undetermined hexose, and the 223-Da component representing an unknown glycan unit (54). An associated peptide fragment was unable to be identified effectively within this extract. This second glycan entity resembles the hexasaccharide moiety that is O-linked to the protein DsbA and has been shown to require the same glycosylation locus as the CLC (60).

Some of the properties of the CLC resemble those of a bacterial surface layer (S-layer), which is a self-assembled monomolecular crystalline array of proteinaceous subunits (61). The proteins in an S-layer are commonly glycosylated and water insoluble due to the high proportion of acidic and hydrophobic amino acids present (62, 63). Coverage of the cell surface with the S-layer varies during different phases of growth (63) and provides a protective function similar to that of a capsule (61, 64). Many of these properties are also common to the CLC. However, S-layers are not common in Gram-negative bacteria (*Campylobacter* is an exception) (65), and S-layers are predominately composed of a single glycoprotein subunit of large molecular size (up to 200-kDa [66]). Although the CLC also contains a large-molecular-size component (likely an aggregate), it appears to be composed of a large number of distinct proteins and glycoproteins, of which many, if not all, are also present in the outer membrane (54).

Genetic Machinery

Two glycosylation or polysaccharide biosynthesis loci have been identified within the *F. tularensis* genome. One of these two loci is the previously mentioned O-antigen locus, which spans open reading frames (ORFs) FTT_1447 through FTT_1464 and is involved in production of type A O-antigen subunits for LPS and the O-antigen capsule. The second locus spans ORFs FTT_0789 to FTT_0800 in *F. tularensis* type A (Table 2 and Fig. 3) and contains proteins with predicted glycosylation functions. Homologs of this gene cluster have been identified in the other subspecies of *F. tularensis*, supporting the hypothesis that this is a conserved mechanism with a possible role in virulence (24, 54, 60, 67). Interruption of two genes within the locus, FTT_0791 and FTT_0798, in SchuS4 interrupts glycosylation of the protein DsbA, an essential virulence factor and possible

lipoprotein, but does not affect O-antigen production (60). Conversely, interruption of the O-antigen locus does not affect the presence of the glycoform on DsbA, verifying that these loci are involved in two separate glycosylation processes (60). The specific glycosylation moiety observed in this study is similar to the hexasaccharide that has been identified in soluble portions of the CLC (60).

An LVS with a double mutation knocking out FTL_1432 and FTL_1431 (homologous genes to FTT_0789 and FTT_0790 in SchuS4, respectively) was created specifically to study the effect of this glycosylation locus on the CLC (24). Interruption of these two genes significantly reduces the carbohydrate present in CLC extracts, and the CLC is no longer evident around the cells by electron microscopy (24). A similar mutation in SchuS4 results in the same effect, with the level of protein in the CLC remaining similar to that of the parent (54). However, the CLC observed on these mutants is significantly lower in concentration than the CLC on the parent, and the CLC does not associate closely with the bacterial cells (54). This genetic locus appears to function primarily, if not exclusively, in glycosylation modifications of *F. tularensis* CLC proteins.

The genes required for export of the CLC have not been defined. Genes have been identified in *F. novicida* that dramatically decrease OMV/T production or export (58, 68). These genes possibly encode proteins involved in carbon metabolism, lipoic acid biosynthesis, and a cytoplasmic membrane protein (68). Each of these genes (FTN_0337 [*fumA*], FTN_1333 [*tktA*], FTN_0908, and FTN_1037) have homologs in the more virulent *F. tularensis* subspecies that are greater than 94% identical. However, the proposed functions of these similar genes are based on bioinformatic analysis and have not been confirmed experimentally. If the CLC is in part OMV/Ts, then mutagenesis of these genes theoretically should reduce the production of the CLC. Analysis of CLC production of such mutants has not been conducted.

Role in Virulence

Gene knockouts of CLC glycosylation has shed some light on the role of the CLC as a virulence factor. Due to the heterogeneous nature of the CLC, a genetic knockout abolishing the entire presence of the CLC would not be possible. Removing glycosylation of the CLC, though, is possible, as previously mentioned, by targeting two glycosyltransferases within the CLC glycosylation locus. Interruption of CLC glycosylation attenuates LVS in the mouse model (24). Nonetheless, *in vitro* this glycosylation mutant (LVS Δ1423/1422) is not sensitive to complement-mediated killing and is able to replicate within a macrophage cell line (24). However, immunization of mice with this attenuated mutant or with LVS CLC conjugated to an immunogenic protein is highly protective against a high-dose LVS challenge (24, 54).

Unfortunately, the same results were not obtained with the more virulent type A strain SchuS4. Type A CLC immunization does not protect mice against a high-dose intranasal challenge with *F. tularensis* SchuS4, nor is strain SchuS4 attenuated following mutagenesis of the same genes that attenuate LVS (SchuS4 Δ0789/799) although a similar loss of CLC glycosylation occurs (54). Subunit vaccines against tularemia do not provide adequate protection against a virulent high-dose challenge as the subunit vaccine may not stimulate the cell-mediated immunity necessary for protection (69). Due to the intracellular lifestyle of *F. tularensis*, attenuated live strains are more desirable as they induce a more protective T-cell immunity. This variation in attenuation of virulence highlights the difference between *F. tularensis* LVS and SchuS4, at least as it pertains to pathogenesis studies in mice. Although LVS is still virulent for mice, the strain is of low virulence for humans and is less virulent for mice than *F. tularensis* SchuS4. Due to the much greater virulence of type A strains than that of LVS, interrupting CLC glycosylation in SchuS4 may be inadequate to render the strain attenuated in the mouse model. Further testing of these strains in an animal model whose susceptibility to *F. tularensis* is more similar to that of humans, such as the Fischer 344 rat (70), may be warranted.

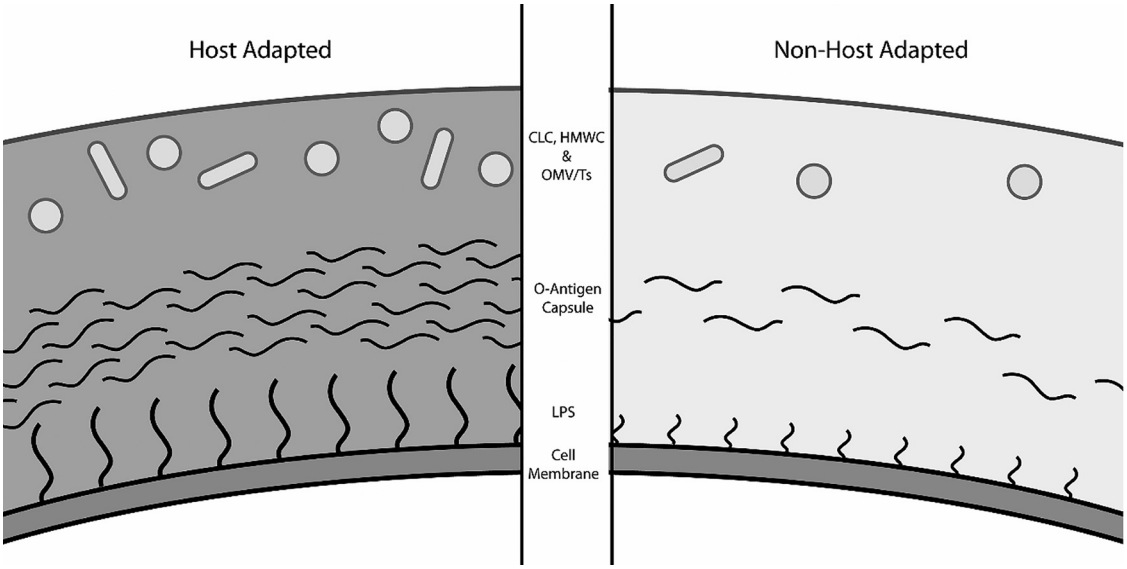

FIG 5 Effect of growth conditions on the extracellular carbohydrates of *F. tularensis* and *F. novicida*. *Francisella* spp. respond to many environmental cues, including free-amino-acid concentrations. The availability of free amino acids for bacterial cells signals a change in the extracellular carbohydrate profile of *F. tularensis* or *F. novicida* or both that affects the LPS, the O-antigen capsule, the CLC, the HMW carbohydrate (HMWC), and the OMV/Ts. Host-adapted *Francisella* bacteria that live extracellularly within the mammalian host live in an environment of low free amino acids, similar to the culture medium BHI broth. These host-adapted bacteria produce longer O-antigen polymers on the LPS, produce more O-antigen capsule, produce a higher concentration of OMV/Ts, and produce greater amounts of CLC or HMWC than non-host-adapted bacteria (grown in amino acid-rich environments, such as the culture medium tryptic soy or Mueller-Hinton broth). *Francisella* spp. grown in tryptic soy broth or Mueller-Hinton broth produce far fewer OMV/Ts, less O-antigen capsule, shorter O-antigen polymers on the LPS, and less CLC.

EFFECT OF GROWTH CONDITIONS

F. tularensis is able to persist in both the intracellular and the extracellular milieus of its mammalian host. However, *F. tularensis* can also survive in the environment, such as in amoebae, arthropod vectors, and, likely, other sources. To adapt to such ecological changes, *F. tularensis* has developed mechanisms to sense changes in its environment and subsequently alter gene expression to adapt to nutritionally variable conditions. The ability of *F. tularensis* to adapt to the host may in part be due to recognizing changes in the amino acid and free-iron concentrations within the host. *F. tularensis* likely encounters environments deprived of amino acids or free iron extracellularly within the host (50, 51, 68). Production of both CLC and OMV/Ts increases in a medium with lower concentrations of free amino acids (such as BHI broth compared to MH broth) (54, 58, 68), as represented schematically in Fig. 5.

Culture of *F. tularensis* in BHI broth better resembles *F. tularensis* found *in vivo* (host-adapted *F. tularensis*) than bacteria cultured in some other medium (49–51). These BHI broth-grown *F. tularensis* cells produce longer LPS O-antigen polymers and a higher concentration of O-antigen capsule, and they produce the HMW carbohydrate that is not present in MH broth-grown bacteria (51) (Fig. 5). BHI broth-grown *F. tularensis* also binds fewer antibodies targeting outer membrane components than MH broth-grown *F. tularensis* (51). These results indicate that the outer membrane is not easily accessible in host-adapted *F. tularensis*, most likely due to upregulation of extracellular capsules, which fits the hypothesis that the *F. tularensis* capsules contribute to the ability of the bacteria to evade the immune response. Supplementing BHI medium with amino acids or using a medium containing high concentrations of amino acids, such as MH broth, represses this host-adapted phenotype (50, 51, 71).

Expression of the CLC is similarly affected by the growth medium. BHI broth- and CDM-grown *F. tularensis* bacteria produce greater amounts of CLC than MH broth-grown *F. tularensis* (54). This difference in CLC production may also be related to differences in the amino acid concentration of the medium or to the presence of

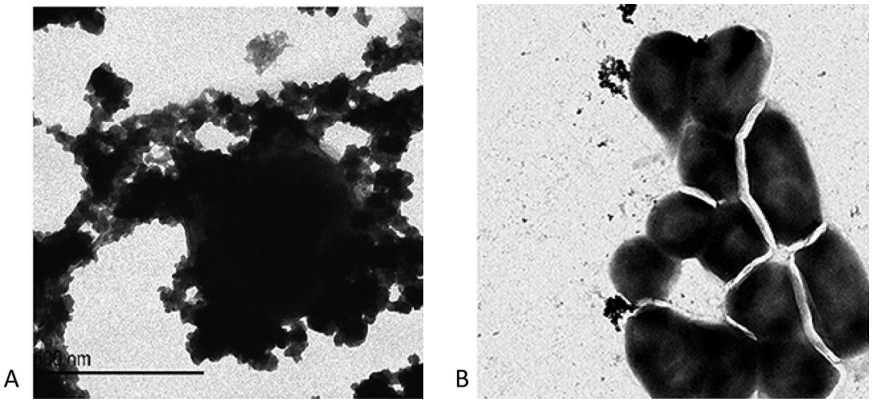

FIG 6 Electron micrographs depicting the *F. novicida* CLC. *F. novicida* produces a CLC similar to the CLC of type A strains when grown under similar culture conditions. The CLC is evident as a granular material surrounding the cells (A). The glycosylation-deficient mutant *F. novicida* Δ1212–1218 produces reduced amounts of this material, and the material present is usually not as adherent to cells (B). (Adapted from reference 67.)

spermidine in CDM but has not been directly assessed. The production of OMV/Ts has also been shown to be affected directly by the concentration of free amino acids. Medium that is deprived of free amino acids, such as BHI broth, leads to increased production of OMV/Ts by *F. novicida*, which is then repressed by the addition of amino acids to the medium (68).

THE *capBCA* LOCUS

Su et al. (72) described a *capBCA* locus in *F. tularensis* LVS that is required for virulence in a mouse respiratory disease model. The CapBC proteins in *F. tularensis* have low (38% and 29%, respectively) amino acid sequence homology to CapBC in *Bacillus anthracis*, which represent two of the five proteins required for synthesis of the poly-γ-D-glutamic acid (PGA) capsule. However, there is no homology between CapA of LVS with CapA of *B. anthracis*. Subsequent studies by these authors showed that mutagenesis of *capB* alone is sufficient to attenuate the bacteria, that the *capBCA* locus is required for intracellular growth of type A strain SchuS4 (73), and that *capBCA* is required for phagosome escape, for inhibition of phagosome maturation, or both. However, we (74) along with Su et al. (73) have found no chemical evidence of the presence of PGA on *F. tularensis* although a glucan exopolysaccharide associated with biofilm development has been identified (74).

THE SIMILAR, BUT DISTINCT, POLYSACCHARIDES OF *F. NOVICIDA*

F. novicida is commonly used as a model organism for pathogenesis studies in place of *F. tularensis*. *F. novicida* is not particularly virulent for humans who are not immunocompromised but is highly virulent for mice. Unlike LVS, though, *F. novicida* does not contain any mutations that may contribute to attenuation. However, as expected, there are similarities but important differences between these species when their capsules and polysaccharides are compared. *F. novicida* has been reported to be unencapsulated (75). Recently, we showed that *F. novicida* produces a CLC similar to that of the more virulent *F. tularensis* when grown in a similar manner on chemically defined medium (24, 67) (Fig. 6A). The carbohydrate contains the same sugars identified in the CLC of LVS (mannose, glucose, and galactose), and interruption of a similar *F. novicida* putative glycosylation locus (FTN_1211 to FTN_1221) leads to the abolishment of that carbohydrate and reduction in the visual presence of CLC by electron microscopy (67) (Fig. 6B). This glycosylation mutant, similar to the LVS glycosylation mutant, is attenuated in the mouse model and provides partial protection against a virulent intranasal challenge (67). Therefore, production and glycosylation of the CLC appear to be a conserved mechanism of *Francisella* subspecies. Differences in the genetic loci responsible for CLC

glycosylation do exist between *F. tularensis* and *F. novicida* and may account for differences in their CLCs' contribution to virulence in *F. novicida* compared to that of the more virulent type A subspecies (Table 2). However, further analysis of the protein component of the *F. novicida* CLC is necessary to establish if proteins similar to those in the more virulent subspecies are present. Analysis of *F. novicida* CLC proteins would also allow for a more direct comparison between the CLC and previously characterized *F. novicida* OMV/T proteins.

Although the presence of a CLC produced by *F. novicida* has been confirmed (67), the presence of an *F. novicida* O-antigen capsule has yet to be determined. MAb 11B7 to the type A/B O-antigen capsule does not react with *F. novicida* strain U112, as expected because *F. novicida* does not produce an O antigen with an identical structure to the O antigens of type A and B strains (23). *F. novicida* U112 also does not react with LPS O-antigen MAb FB11 or with other antibodies that bind the LPS O antigen or O-antigen capsule of type A and type B strains (23). Genetic and compositional analysis of the *F. novicida* O antigen highlights major differences between the *F. novicida* O antigen and the type A and type B O antigens. Although similar, the *F. novicida* O-antigen locus responsible for construction of the tetrasaccharide subunits contains six genes unique to *F. novicida* and does not contain several genes unique to type A and type B strains (76–79) (Table 1). Compositional analysis confirms that the *F. novicida* O-antigen tetrasaccharide subunit is distinct from the O-antigen tetrasaccharide subunits of type A and B strains (20, 79). These structural differences have already been shown to affect the immunogenic properties of *F. novicida in vivo* compared to those of type A and B strains (7, 80, 81). Therefore, it is possible that *F. novicida* produces an *F. novicida*-specific O-antigen capsule that cannot be detected with antibodies to type A/B O-antigen capsule due to differences in their compositional structures.

CONCLUSIONS

Collectively, *F. tularensis* produces two separate extracellular components that function similarly to a traditional capsule. The O-antigen capsule is composed of a polysaccharide similar to the O antigen of the LPS and shares a similar biosynthetic pathway. Strains that lack the O antigen, both the capsule and the LPS, are typically serum sensitive and are attenuated *in vivo*. However, the relative contribution of the O-antigen capsule and the LPS O antigen to serum resistance and virulence is, at this time, unknown. The CLC is a heterogeneous group of proteins, many of which are glycosylated by either a repeating 420-Da glycan subunit or a previously described hexasaccharide associated with glycosylation. The locus responsible for glycosylation of the CLC is a distinct locus from the O-antigen glycosylation locus. The exact nature of the CLC has not been defined but may be (at least in part) composed of OMV/Ts as the appearance and the proteins identified in OMV/Ts are highly similar to the appearance and proteins identified in CLC extracts. The HMW carbohydrate present in host-adapted *F. tularensis* may represent a high-molecular-size aggregate of the CLC or could be a third extracellular capsule-like component. Optimal expression of both the O antigen and the CLC appears to be influenced by growth conditions that simulate growth conditions in the host. However, the exact role that either capsule plays in the virulence of *F. tularensis* has not been fully elucidated as there are overlaps between the functions of the O-antigen capsule and the LPS and the functions of the CLC and OMV/Ts. Further studies are necessary to delineate the contributions of each capsule to the pathogenesis and virulence of *F. tularensis*.

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